

Specific labeling of newly synthesized lipopolysaccharide via metabolic incorporation of azido-galactose

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ABSTRACT

Gram-negative bacteria possess an asymmetric outer membrane (OM) primarily composed of lipopolysaccharides (LPS) on the outer leaflet and phospholipids on the inner leaflet. The outer membrane functions as an effective permeability barrier to compounds such as antibiotics. Studying LPS biosynthesis is therefore helpful to explore novel strategies for new antibiotic development. Metabolic glycan labeling of the bacterial surface has emerged as a powerful method to investigate LPS biosynthesis. However, the previously reported methods of labeling LPS are based on radioactivity or difficult-to-produce analogs of bacterial sugars. In this study, we report on the incorporation of azido galactose into the LPS of the Gram-negative bacteria *Escherichia coli* and *Salmonella typhi* via metabolic labeling. As a common sugar analog, azido galactose successfully labeled both O-antigen and core of *Salmonella* LPS, but not *E. coli* LPS. This labeling of *Salmonella* LPS, as shown by SDS-PAGE analysis and fluorescence microscopy, differs from the previously reported labeling of either O-antigen or core of LPS. Our findings are useful for studying LPS biogenesis pathways in Gram-negative bacteria like *Salmonella*. In addition, our approach is helpful for screening for agents that target LPS biosynthesis as it allows for the detection of newly synthesized LPS that appears in the OM. Furthermore, this approach may also aid in isolating chemically modified LPS for vaccine development or immunotherapy.

1. Introduction

Metabolic glycoengineering (MGE) has been developed as a powerful approach to insert non-natural monosaccharide into glycan structures on the cell surface [1] or secreted glycoproteins [2]. The strategy depends on a modified monosaccharide analog containing a bioorthogonal chemical reporter that hijacks the glycan biosynthetic pathways such that it is installed into the glycans of cells, in place of the natural monosaccharide. Following the incorporation, these reporters can be tagged with a fluorescent probe through a bioorthogonal (click chemistry) reaction, enabling visualization or analysis of the labeled

biological molecules and related cellular structures by, for instance, fluorescence microscopy or in-gel fluorescence imaging. Metabolic labeling of glycans has been widely used for studying eukaryotic systems [3–11]. In contrast, only a few of these approaches have been reported for bacteria [12–19].

Gram-negative bacteria are covered by an asymmetric outer membrane that consists of a dense lipopolysaccharide (LPS) layer as its outer leaflet (Fig. 1A). In most cases, LPS is essential for bacterial viability as is also the case for *Escherichia coli* (*E. coli*) [22]. The special structure of LPS functions as a permeability barrier for hindering small, hydrophobic molecules that can otherwise cross phospholipid bilayers, which renders

Abbreviations: *E. coli*, *Escherichia coli*; GalE, UDP-galactose-4-epimerase; GalTs, galactosyltransferases; Hep, glycerol- α -D-manno-heptopyranose; KDO, 3-deoxy- α -D-manno-oct-2-ulopyranosonic acid; LPS, lipopolysaccharides; MGE, Metabolic glycoengineering; NMR, nuclear magnetic resonance; OM, outer membrane; TMS, tetramethylsilane; UDP-galactose, uridine diphosphate galactose.

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Gram-negative bacteria innately resistant to many antimicrobial compounds. LPS is made up of several types of different sugars and fatty acids and its structure can be divided into three regions: a glyco-phospholipid moiety called lipid A, a core oligosaccharide, and an O-antigen. The lipid A structure corresponds to a bis-phosphorylated β -1-6 glucosamine disaccharide, to which depending on the bacterium up to seven acyl chains are attached via ester or amide linkages. The inner core is typically composed of 3-deoxy- α -D-manno-oct-2-ulopyranosonic acid (KDO) and heptose sugars. KDO is a near-universal component of LPS, forming an α -linkage with the glucosamine disaccharide of lipid A in nearly all instances. The outer core is characterized by common hexose sugars such as glucose, galactose, *N*-acetyl galactosamine, and *N*-acetyl glucosamine. The O-antigen is only exposed on the surface of

bacteria with a smooth phenotype and the LPS is called S-LPS. Some bacteria have a rough morphology and their LPS is called R-LPS, which is deprived of O-antigen, e.g., in the *E. coli* K12 strain. While the O-antigen region is extremely variable, the lipid A and core oligosaccharide regions are much more limited in variation. In *E. coli*, the inner core is composed of a conserved structural element of KDO and L-glycero- α -D-manno-heptopyranose (Hep), which also can contain phosphoethanolamine and phosphate residues. Five unique outer core structures have so far been determined in *E. coli* (R1–R4 and K12) [23]. The different sugars in LPS are linked uniquely when comparing different bacteria. For example, in some *Salmonella* strains, galactose is present in the LPS core as an α -1,3-linked backbone sugar and an α -1,6-linked branch substituent (Fig. 1B) [20,24]. Galactose is only present as the α -1,6-

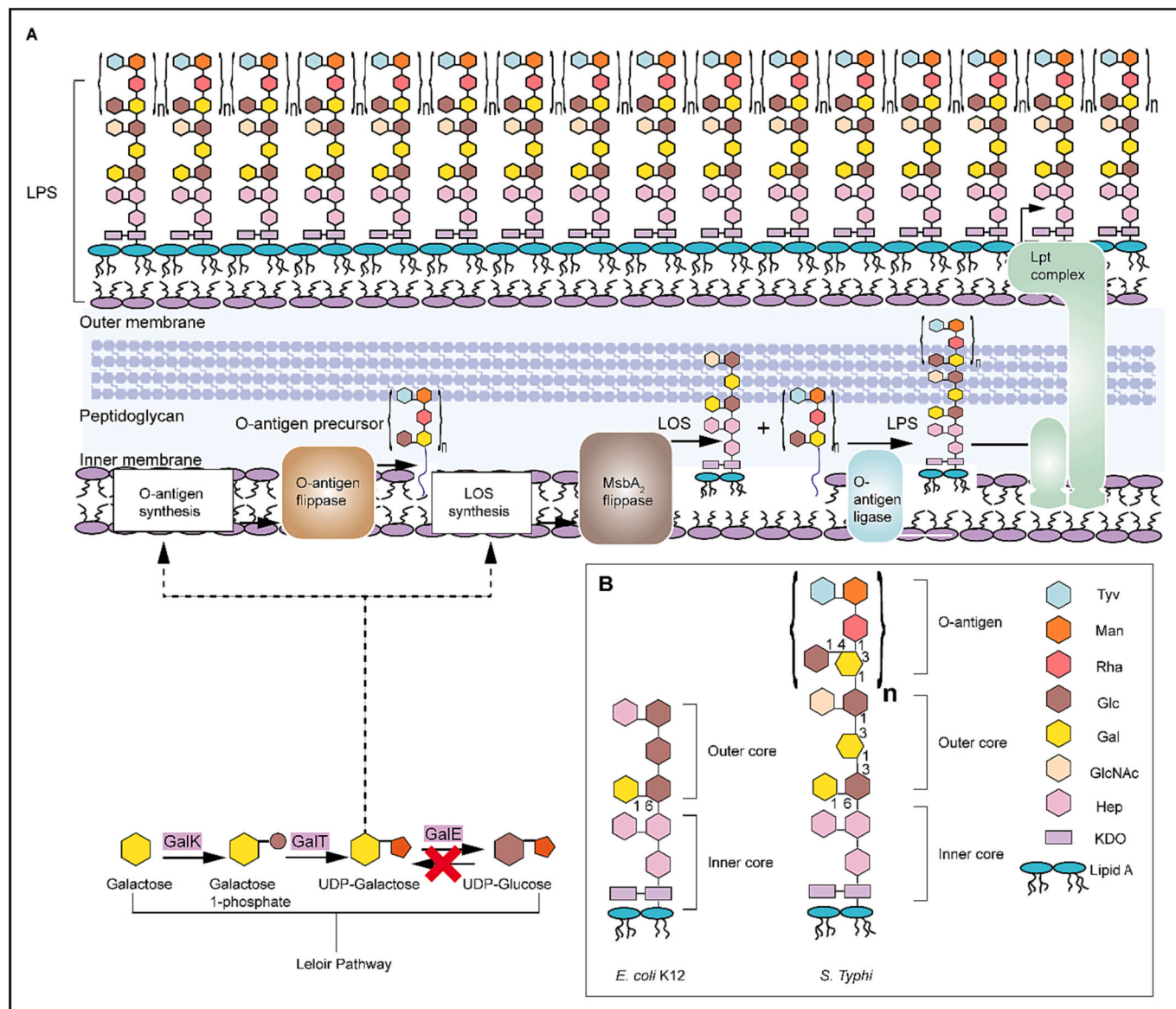


Fig. 1. Schematic of biosynthesis of LPS in serovar Typhi and LPS structure of *E. coli* K12 and serovar Typhi [20]. **A)** Lipopolysaccharide (LPS) biogenesis in *Salmonella enterica serovar Typhi*. In brief, the O-antigen repeating unit and core domains of LPS are assembled and synthesized at the cytoplasmic interface of the inner membrane. Core domains are flipped by MsbA₂, and O-antigen precursors are flipped by O-antigen flippases across the inner membrane, then the O-antigen is attached to the core domain (LOS) at the periplasmic side of the inner membrane. Finally, the Lpt complex transports LPS from the inner membrane to the surface of the outer membrane. UDP-galactose is the galactose donor in both the O-antigen repeat unit and core domain synthesis processes. For *gal E* mutants, UDP-galactose cannot be obtained by converting UDP-glucose due to a lack of the Gal E enzyme. Therefore, in the case of *S. typhi*, coupling of the O-antigen to the core domain is dependent on the presence of externally provided galactose. **B)** The reported structures of the core domains and O antigen repeat unit of *E. coli* K12 and *S. typhi*. In *Salmonella*, galactose is present in the LPS core as an α -1, 3-linked backbone sugar and an α -1, 6-linked branch substituent, while galactose is only as the α -1, 6-linked branch substituent in LPS core of *E. coli* K-12. The number represents the positions of linkage on sugars. (Figure adapted from Simpson and Trent) [21].

linked branch substituent in the LPS core of *E. coli* K-12 [24,25], while in *E. coli* with the R3 core type, galactose is linked as α -1,3-linked backbone sugar in the LPS core [24]. Metabolic glycoengineering may provide a useful tool to i) decipher the process of LPS biogenesis in bacteria and ii) specifically detect bacteria [26–29]. However, the labeling of lipopolysaccharide (LPS) of Gram-negative bacteria was so far limited to special monosaccharides, i.e. KDO [14,16] in the core of the LPS or rare oligosaccharide analogs [15,17–19] in the O-antigen part.

Galactose, one of the common monosaccharides that is present in both the core and O-antigen of LPS, is a very attractive candidate for labeling LPS. The main pathway of galactose metabolism in bacteria is the Leloir pathway (Fig. 1) [30]. In this pathway, galactose is converted to galactose-1-phosphate, which is subsequently transformed to uridine diphosphate galactose (UDP-galactose). UDP-galactose is a universal donor for galactosyltransferases (GalTs) that mediate the addition of galactose to LPS in bacteria. Another important metabolic route to UDP-galactose is its synthesis from UDP-glucose by UDP-galactose-4-epimerase (GalE), an enzyme that usually works in both directions. Deletion of the GalE gene in bacteria ($\Delta galE$ mutant) blocks this conversion between UDP-glucose and UDP-galactose. In these bacteria, the one possible metabolic flux for UDP-galactose is as a donor to LPS. Consequently, the LPS of $\Delta galE$ *S. typhi* strains is truncated when grown in the absence of galactose, resembling deep rough phenotypes with only lipid A, an inner core, and an incomplete outer core (Fig. 1). Upon the addition of galactose to the growth medium, the synthesis of complete LPS is restored. This phenomenon allowed the utilization of radiolabeled galactose to follow newly synthesized LPS in *galE*-deficient bacteria [25]. Here, we first explored the possibility of azido galactose incorporation into the LPS of *galE*-deficient *E. coli* strains. However, apparent substrate specificities within the metabolic pathway of galactose in this bacterium hindered this approach. Nonetheless, we could demonstrate the incorporation of azido galactose in both the core and O-antigen of newly synthesized LPS of the *galE*-deficient strains of *Salmonella enterica* serovar *Typhi* (Ty21a). The newly synthesized LPS could be fluorescently labeled in live bacteria, and the resulting images provided evidence that the newly synthesized LPS is also inserted into the poles of the bacteria. This is an interesting finding that differs from the previous results that could only show labeled LPS insertion mid-cell [31–34].

2. Experimental procedures

2.1. General information

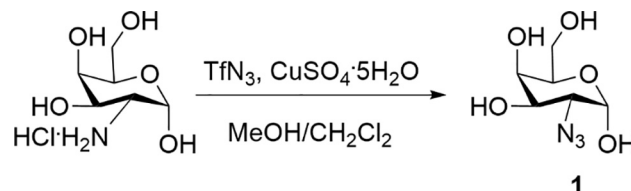
All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. All non-aqueous reactions were performed in dry glassware under a slight positive pressure of nitrogen unless otherwise noted.

All NMR spectra were recorded at 400 MHz on an Agilent NMR machine (400-MR spectrometer) with chemical shifts reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS). ^1H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet), number of protons, and coupling constants (J) in Hertz (Hz). Mass spectrometry (MS) analysis was performed using an ESI-MS (Thermo Finnigan, LCQDECA XP) instrument. The fluorescent scanning SDS-PAGE gels were performed on a GE Amersham™* Imager 600 series.

The avirulent *Salmonella typhimurium* strain SF1195 is identical to LT2 SL 848 [35] and the *galE* deficient strain Ty 21a of *Salmonella typhi* is constructed from strain Ty 2 [36], which both were generous gifts from Dr. J.P. van Ulsen of Amsterdam Institute of Molecular and Life Sciences and Dr. B. Appelmek of the Department of Medical Microbiology and Infection Control at Amsterdam University Medical Center. *E. coli* K12 strains included wild types (W3110), a *galE*-deficient strain from the Keio collection of *E. coli* (JW0742), and the *galE*-deficient strain of *E. coli* CWG1170, which was generously provided by the Whitfield lab [37]. The plasmid of GalK from *Streptococcus pneumoniae* TIGR4 (*GalKS*_{pe4})

was a gift from Prof. Min Chen from the State Key Laboratory of Microbial Technology, National Glycoengineering Research Center, Shandong University, China. All the strains were grown in lysogeny broth (LB) or M9 minimal medium while shaking (250 rpm) at 37 °C unless otherwise stated.

2.2. Synthesis of azido galactose

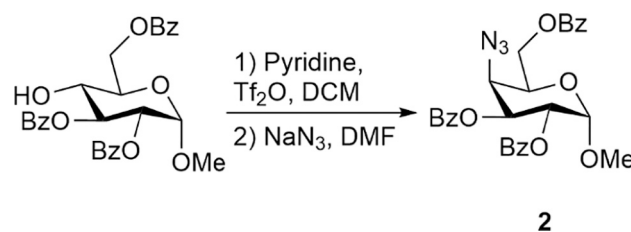


2.2.1. 2-Azido-2-deoxy-D-galactose (1)

2-azido-2-deoxy-D-galactose was prepared from D-galactosamine hydrochloride according to the previously reported paper with some modifications [38,39]. In detail, 2-amino-2-deoxy galactose hydrochloride (0.65 g, 3 mmol) was suspended in CH_2Cl_2 (2 mL) in a 50 mL round-bottomed flask. To the mixture were added Et_3N (0.90 g, 1.25 mL, 9 mmol) and a solution of CuSO_4 (24 mg, 0.15 mmol, in 0.5 mL water). Subsequently, a freshly prepared solution of trifluoromethanesulfonic anhydride (TfN_3 , 9 mmol) in CH_2Cl_2 (6 mL) was added to the above reaction mixture and the solution was brought to homogeneity by adding MeOH (2 mL). The reaction solution was stirred at room temperature for 17 h. Then, the reaction mixture was poured into saturated aqueous NaHCO_3 (30 mL) and stirred for 15 min, and the mixture was extracted with CH_2Cl_2 three times (3×30 mL). The combined organic phases were filtered through Celite, washed with brine (30 mL), dried with anhydrous MgSO_4 , filtered, and concentrated. The residue was purified with flash silica gel chromatography (ethyl acetate: MeOH = 9:1), yielding a light off-white solid (485 mg, 79 %).

^1H NMR (400 MHz, D_2O) δ 5.22 (d, J = 3.9 Hz, 0.4H), 4.57 (d, J = 7.9 Hz, 0.6H), 4.22–3.88 (m, 2.6H), 3.76 (m, 3H), 3.48 (dd, J = 9.7, 8.0 Hz, 0.4H).

ESI-MS m/z calcd for $[\text{C}_6\text{H}_{11}\text{N}_3\text{O}_5\text{Na}]^+ ([\text{M} + \text{Na}]^+)$ 228.06, found 228.43.

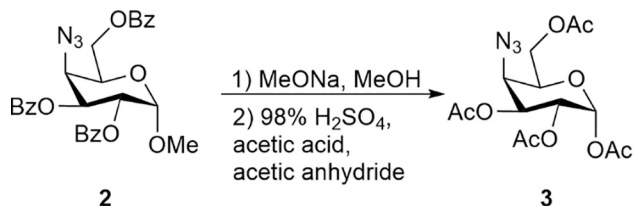


2.2.2. 4-Azido-2, 3, 6-tri-O-benzoyl-4-deoxy-D-galactopyranoside (2)

Methyl 2, 3, 6-tri-O-benzoyl-D-glucopyranoside (2.04 g, 4 mmol), and pyridine (1.03 mL, 12.8 mmol) were dissolved in DCM (50 mL). The reaction mixture was cooled to -10 °C in a calcium chloride-ice bath. Subsequently, triflic anhydride (0.89 mL, 5.3 mmol) was added to the reaction solution and the solution became light pink after being stirred for 30 min in an ice bath and then for 2 h at room temperature. The reaction solution was diluted with ethyl acetate (EtOAc , 150 mL) and washed with water (150 mL). The aqueous phase was extracted with EtOAc (150 mL) once again. The combined organic phases were washed with water (2×150 mL), a saturated NaCl solution (100 mL), dried with MgSO_4 , and then filtered and concentrated. The resulting residue was dissolved in DMF (15 mL) and stirred with NaN_3 (1.3 g, 20 mmol) at 60 °C overnight. The reaction solution was diluted with EtOAc (50 mL)

and washed with water (50 mL). The aqueous phase was extracted with EtOAc (50 mL) once again. The combined organic phases were washed with water twice (2×50 mL), a saturated NaCl solution (50 mL), dried with MgSO_4 , filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane: EtOAc = 3:1), giving 1.21 g of a foam-like product (yield, 57 %).

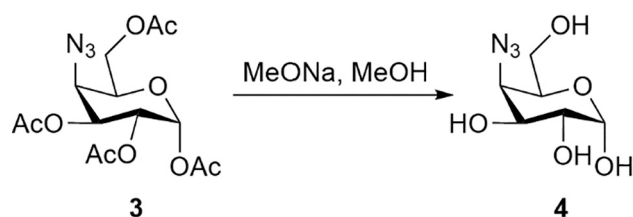
^1H NMR (400 MHz, CDCl_3) δ 8.05 (dd, $J = 10.9, 4.1$ Hz, 4H), 8.01–7.95 (m, 2H), 7.62–7.34 (m, 9H), 5.96 (dd, $J = 10.6, 3.7$ Hz, 1H), 5.61 (dd, $J = 10.6, 3.7$ Hz, 1H), 5.19 (d, $J = 3.7$ Hz, 1H), 4.62–4.49 (m, 2H), 4.44–4.33 (m, 2H), 3.43 (s, 3H).



2.2.3. Tetra-acetate 4-azido galactose (3) 2

(1.03 g, 1.9 mmol) was dissolved in 15 mL methanol, treated with MeONa (1.2 mL, 25 %, 5.4 mmol) solution and stirred overnight at room temperature. The mixture was subsequently neutralized with DOWEX 50WX8–400 ion-exchange resin and the filtrate was concentrated. The residue was dissolved in 4 mL of glacial acetic acid and 4 mL of acetic anhydride. Then, 0.8 mL of concentrated sulfuric acid was added. The mixture was stirred at room temperature overnight. Sodium acetate was added to neutralize the pH to 7. Subsequently, the mixture was poured into ice water (40 mL) and extracted with dichloromethane (3×40 mL). The organic layers were combined and washed with aqueous saturated NaHCO_3 (3×40 mL), aqueous saturated NaCl (40 mL), dried with magnesium sulfate, and evaporated to dryness under vacuum. The residue (brown oil) was purified by silica gel column chromatography (EtOAc: hexane = 1: 1) to obtain a colorless oil product (383 mg, 54 %).

^1H NMR (400 MHz, CDCl_3) δ 6.28 (s, 1H), 5.39–5.34 (m, 2H), 4.22–4.12 (m, 4H), 2.11 (d, $J = 2.3$ Hz, 6H), 2.06 (s, 3H), 1.99 (s, 3H).

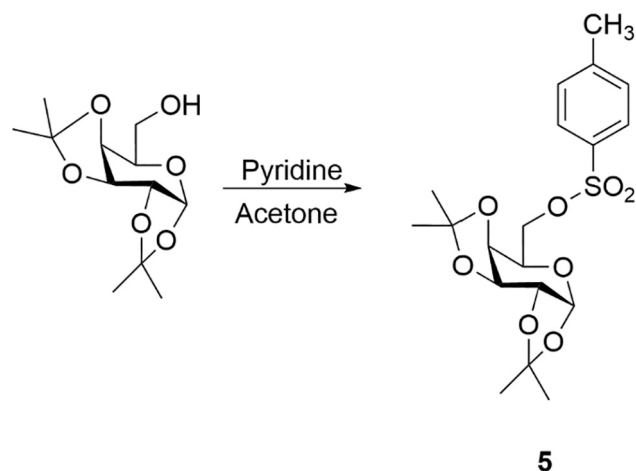


2.2.4. 4-Azido-4-deoxy-D-galactose (4) 3

(383 mg, 1.02 mmol) was dissolved in 10 mL anhydrous MeOH, treated with MeONa (1.2 mL, 25 %, 5.4 mmol) solution, and the mixture was stirred overnight at room temperature. The mixture was subsequently neutralized with DOWEX 50 \times 8–400 ion exchange resin until the pH reached 7. The mixture was filtered, concentrated, and purified by silica gel column chromatography (using CHCl_3 : MeOH = 5:1 as the eluent), giving 56 mg of the product (yield, 27 %).

^1H NMR (400 MHz, D_2O) δ 4.65 (d, $J = 8.1$ Hz, 1H), 3.93 (d, $J = 3.2$ Hz, 1H), 3.77 (d, $J = 4.2$ Hz, 2H), 3.72–3.66 (m, 2H), 3.48 (dd, $J = 10.4, 8.2$ Hz, 1H).

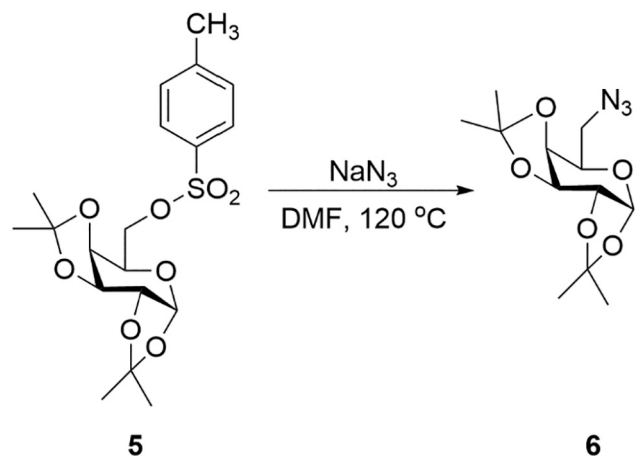
ESI-MS m/z calcd for $[\text{C}_6\text{H}_{11}\text{N}_3\text{O}_5\text{Na}]^+ ([\text{M} + \text{Na}]^+)$ 228.06, found 228.50.



2.2.5. 1, 2: 3, 4-Di-O-isopropylidene-6-O-p-toluenesulfonyl- α -D-galactopyranose (5)

To a solution of diacetone-D-galactose (10.5 g, 40 mmol) and pyridine (13 mL, 161 mmol) in a 100 mL branch flask bottle with 12 mL acetone in an ice-water bath was added 4-toluenesulfonyl chloride (9.4 g, 49 mmol) in portions. Then, the reaction was kept stirring at room temperature for 17 h under nitrogen flow. To the solution was added 40 mL of water, which led to a yellow precipitate. The precipitate was filtered and washed with water twice (2×80 mL). The sticky and yellow solid was recrystallized in 30 mL isopropanol (heating the suspension until the solid totally dissolved in the isopropanol, and then cooling down the system to room temperature) yielding a white solid (10.2 g, 62 %).

^1H NMR (400 MHz, CDCl_3) δ 7.80–7.78 (m, 2H), 7.32–7.30 (m, 2H), 5.43 (d, $J = 5.0$ Hz, 1H), 4.57 (dd, $J = 7.9, 2.5$ Hz, 1H), 4.27 (dd, $J = 5.0, 2.5$ Hz, 1H), 4.21–4.16 (m, 2H), 4.10–4.00 (m, 2H), 2.42 (s, 3H), 1.48 (s, 3H), 1.33 (s, 3H), 1.29 (s, 3H), 1.26 (s, 3H).

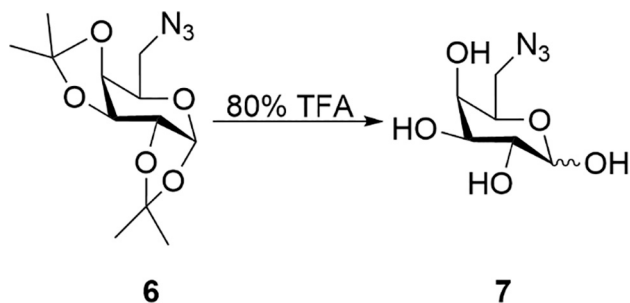


2.2.6. 6-Azido-6-deoxy-1, 2: 3, 4-di-O-isopropylidene- α -D-galactopyranose (6)

To a 250 mL flask was added 5 (4.4 g, 10.6 mmol), sodium azide (10.3 g, 0.158 mol), and DMF (100 mL). The reaction mixture was heated up to 135 °C and stirred for 48 h. The reaction mixture was cooled to room temperature, diluted with water (150 mL), and extracted with ethyl acetate three times (3×150 mL). The combined organic phases were washed with water three times (3×200 mL), a saturated NaHCO_3 solution (200 mL), again washed with water three times (3×200 mL), dried with MgSO_4 , and concentrated, giving a clear syrup. The

syrup was purified with silica column chromatography (hexane: EtOAc = 7: 1), giving a clear sticky syrup (2.6 g, 87 %).

^1H NMR (400 MHz, CDCl_3) δ 5.54 (d, J = 5.0 Hz, 1H), 4.62 (dd, J = 7.9, 2.5 Hz, 1H), 4.33 (dd, J = 5.0, 2.5 Hz, 1H), 4.19 (dd, J = 7.9, 2.0 Hz, 1H), 3.91 (ddd, J = 7.5, 5.4, 1.9 Hz, 1H), 3.50 (dd, J = 12.7, 7.8 Hz, 1H), 3.35 (dd, J = 12.7, 5.3 Hz, 1H), 1.54 (s, 3H), 1.45 (s, 3H), 1.33 (d, J = 2.4 Hz, 6H).



2.2.7. 6-Azido-6-deoxy-D-galactose (7)

To a 100 mL round-bottomed flask was added 6 (2.28 g, 8 mmol), and trifluoroacetic acid (80 %, 64 mL). The reaction was stirred at room temperature for 2 h and evaporated using a toluene azeotrope, yielding a white powder (1.6 g, 98 %).

^1H NMR (400 MHz, D_2O) δ 5.19 (d, J = 3.7 Hz, 1H), 4.11 (dd, J = 8.4, 4.6 Hz, 1H), 3.90–3.85 (m, 1H), 3.75 (ddd, J = 25.3, 10.3, 3.5 Hz, 2H), 3.42 (m, J = 13.0, 6.6 Hz, 2H).

ESI-MS m/z calcd for $[\text{C}_6\text{H}_{11}\text{N}_3\text{O}_5\text{Na}]^+ ([\text{M} + \text{Na}]^+)$ 228.06, found 228.00.

2.3. SDS-PAGE analysis and silver staining of LPS

A total of 200 μL bacteria suspension ($\text{OD}_{600\text{nm}} = 1$) was pelleted and resuspended in 10 μL 2 \times Laemmli sample buffer, then the samples were boiled for 10 min and allowed to cool to room temperature. Subsequently, 4 μL of Tris-HCl buffer (0.25 M, pH 6.8), 4 μL of 10 % EDTA solution (in Milli-Q water), and 2 μL of Proteinase K (2.5 mg mL^{-1}) in Laemmli buffer were added and samples were incubated with shaking at 37 $^\circ\text{C}$ for 3 h. Finally, the samples (4 μL) were loaded on an 18 % SDS-PAGE gel, which was run for 1 h at a constant voltage of 80 V and then for 2 h at 120 V.

The LPS was visualized by silver staining as described [40] with some modifications. In detail, the gels were fixed in fresh Milli-Q water containing 30 % ethanol and 10 % acetic acid (100 mL) and then washed with fresh Milli-Q water three times (3×100 mL) for 30 min. Subsequently, the gels were incubated in 0.1 % silver-nitrate solution (100 mL) for 30 min, washed with Milli-Q water (200 mL) once for 10 s, and reduced by formaldehyde (0.2 %) in sodium carbonate (3 %) solution (100 mL) to visualize LPS bands. The visualization process was stopped after 5 min by adding a 1 % acetic acid solution (100 mL), replacing the reduction buffer. The gels were then kept in a 1 % acetic acid solution until imaging.

2.4. LPS remodeling in bacteria

For bacterial cell wall remodeling and labelling, overnight precultured bacteria were inoculated to an $\text{OD}_{600\text{nm}}$ of 0.05 into fresh LB (2 mL) or M9 medium (2 mL) containing azido-galactose and incubated at 37 $^\circ\text{C}$ for 8 h or according to the specific experimental requirements. The azido-galactose variants employed comprised 2-deoxy-2-azido galactose, 4-deoxy-4-azido galactose, and 6-deoxy-6-azido galactose at concentrations of 0.2 %, 2 % or adjusted as necessary based on experimental objectives. A concentration of 0.2 % of unmodified galactose was used as a control. The bacteria cells were then harvested by centrifugation at

4000 $\times g$ for 2 min and subsequently washed three times with 1 mL of PBS. After washing, the bacterial suspension was adapted to an $\text{OD}_{600\text{nm}}$ of 2 in PBS and 1 mL was then used for the click reaction.

2.5. LPS labeling in bacteria

To perform the copper-catalyzed click reaction, 0.5 mL of a washed bacterial suspension at $\text{OD}_{600\text{nm}} = 2$ was centrifuged at 4000 $\times g$ for 2 min. The resulting pellet was then mixed with 25 μL of PBS (pH 7.4), 5 μL of CuSO_4 solution (10 mM), 5 μL of tris-hydroxypropyltriazolymethylamine (THPTA, 10 mM), 5 μL of freshly prepared ascorbate sodium solution (25 mM), and 10 μL either Alexa Fluor 488 alkyne (100 μM) or Atto-550 alkyne (100 μM). This suspension was then incubated at room temperature in the dark for 30 min. Subsequently, the bacteria were washed with 50 μL of PBS six times and resuspended in 50 μL of PBS, and stored at 4 $^\circ\text{C}$ until further analysis.

To perform the copper-free catalyzed click reaction, 0.5 mL of the previously washed bacterial suspension ($\text{OD}_{600\text{nm}} = 2$) was centrifuged at 4000 $\times g$ for 2 min. The resulting pellet was then mixed with 50 μL PBS containing 250 μM DBCO-Cy5. This suspension was incubated in the dark at room temperature for 1 h, washed as described above and resuspended in 50 μL PBS before being prepared for in-gel fluorescence visualization.

2.6. Fluorescence microscopy of the azido-galactose labeled bacteria

First, 200 μL of the remodeled bacteria suspension ($\text{OD}_{600\text{nm}} = 1$) (see above in the remodeling section) was harvested and incubated with the labeling buffer of click chemistry at a total volume of 50 μL as above. After labeling the bacteria via the copper-catalyzed click chemistry with the Alexa Fluor 488-dye, washing, and resuspension in 100 μL PBS, a sample of the bacterial suspension (50 μL) was taken and applied onto a glass slide covered with a flat and thin 1 % agarose pad mixed with PBS buffer (pH 7.4). The glass slide loaded with bacterial cells was immediately transferred to a Leica Fluorescence Inverted Microscope Solution DMI8 equipped with a 100 \times objective. ZEN software was used to collect the data, and the images were analyzed using Image J.

2.7. Time-course of 2-azido-2-deoxy galactose incorporation into LPS

The Ty21a strain was cultured overnight, and a 100-fold diluted sample was prepared in 20 mL of LB medium. The culture was then placed in a shaker at 37 $^\circ\text{C}$ and 250 rpm until it reached an $\text{OD}_{600\text{nm}}$ of 0.5. At this point, 2-azido-2-deoxy galactose was added to the LB medium, making it 0.5 % of the final concentration. Samples were collected at specific time intervals (0 min, 1 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 16 h). Each sample was centrifuged at 4000 $\times g$ for 2 min, and the resulting bacterial pellets were washed three times with PBS buffer. The pellets were then suspended in PBS buffer to achieve a final $\text{OD}_{600\text{nm}}$ of 0.5. For each time interval, 0.5 mL of the bacterial suspension was centrifuged at 4000 $\times g$ for 2 min. To the pellets, 50 μL of PBS containing DBCO-sulfo-Cy5 (250 μM) was added and incubated in the dark at 37 $^\circ\text{C}$ for 1 h. The bacteria were washed six times with 200 μL of PBS and suspended in 200 μL of PBS. The bacteria were then prepared for SDS-PAGE analysis as described above. The SDS-PAGE gel was washed once with water and scanned for 3 min using a GE Amersham™ Imager 600 series at the red channel. The resulting images were analyzed using Image J.

2.8. Metabolomics of galactose in *E. coli*

To overcome the apparent narrow substrate specificity of *E. coli* GalK, the first enzyme of the Leloir pathway [41], we expressed GalK from *Streptococcus pneumoniae* TIGR4 (*GalKSpe4*) in bacteria [42,43]. For this, wild-type *E. coli* strain W3110 and mutant *E. coli* strains CWG1170 (ΔgalE) or JW0742 (ΔgalE), all transformed with the GalK

from *Streptococcus pneumoniae* TIGR4 (*GalKSpe4*), were cultured in LB or M9 medium supplemented with 100 $\mu\text{g mL}^{-1}$ ampicillin and 100 μM IPTG (for induction of *GalK* expression) until an $\text{OD}_{600\text{nm}}$ of 0.6 was reached in the presence or absence of various azido-galactose variants. Subsequently, 1 mL of bacteria was centrifuged at 3000 $\times g$ for 2 min, and the pellet was washed twice with 1 mL of Tris-HCl (50 mM, pH 8.0). The washed bacteria were lysed in 0.4 mL of lysis solution (acetonitrile: methanol: water = 2: 2: 1) while vortexing and the lysed cells were cooled on ice for 10 mins. The samples were centrifuged at 16,000 $\times g$ for 15 min at 4 °C, and the supernatants were collected for LC-MS analysis. LC-MS analysis was performed on an Exactive mass spectrometer (Thermo Scientific) equipped with a Dionex Ultimate 3000 autosampler and pump (Thermo Scientific). Metabolites were identified and quantified using LCquan (Thermo Scientific) and validated by retention time concordance with standards.

3. Results and discussion

3.1. The exploration of galactose and azido-galactose incorporation into LPS of *E. coli*

We initially tested whether the *gal E* deficient strain from the Keio collection of *E. coli* single-gene deletions (JW0742) could incorporate 2-azido-2-deoxy or 6-azido-6-deoxy galactose into its LPS. Although we were able to demonstrate that bacteria incorporated regular galactose into their LPS based on the shift of staining bands on SDS-PAGE (Fig. S1A), we were unable to detect any incorporation of 2-azido-2-deoxy or 6-azido-6-deoxy galactose into LPS, even at a high concentration of 2 %. This may be due to a narrow substrate specificity of *E. coli* *GalK*, the first enzyme of the Leloir pathway (Fig. 1) [41]. To overcome this possible limitation, we expressed *GalK* from *Streptococcus pneumoniae* TIGR4 (*GalKSpe4*) in the JW0742 strain. *GalKSpe4* was shown to have a broader substrate specificity for galactose derivatives with modifications on the C6-position [43]. However, we could not prove the incorporation of 2-azido-2-deoxy nor 6-azido-6-deoxy galactose into LPS of JW0742 expressing *GalKSpe4* even though there seemed to be a slight increase in one of the LPS bands with respect to the control without galactose (Fig. S1B). The bands on the SDS-PAGE were always reproducible, but the signals varied somewhat in intensity between samples. To gauge why these cells failed to incorporate azido-galactose into LPS, we analyzed the metabolism of 6-azido-6-deoxy galactose in JW0742 compared to another *Gal E* mutant strain *E. coli* CWG1170 ($\Delta galE$) and a wild-type *E. coli* strain (W3110), all expressing *GalKSpe4* (Fig. S2). Through metabolomics studies with mass spectrometry, we observed that azido-galactose could be detected in the metabolites of all tested strains, suggesting that all strains were capable of importing the azido-galactose inside the cells in a concentration-dependent way. While minor amounts of azido-galactose 1-phosphate could be observed in all of the strains at the highest azido-galactose concentration (2 %), UDP-azido-galactose could only be observed in the metabolites of JW0742. Based on the Leloir pathway of galactose metabolism, this suggests that i) The galactokinase *GalKSpe4* can use 6-azido-6-deoxy galactose as a substrate, ii) it is important to use bacteria with a *GalE* deletion for this approach (Fig. S2) and iii) that apparently also the substrate specificity of galactose-1-phosphate uridylyltransferase (*GalT*) varies between different strains. In addition, these results also show that failure to incorporate azido-galactose into the LPS of *E. coli* cells is likely caused by the narrow substrate specificity of the enzymes responsible for the incorporation of 6-azido-6-deoxy galactose into their LPS. In *E. coli* K-12, galactose is present in LPS only as the α -1,6-linked branch substituent, making it difficult to detect the incorporation of galactose (Fig. 1) as only a small mass difference is expected to occur upon incorporation of galactose in the LPS. Therefore, we shifted our focus to *Salmonella* strains.

3.2. Metabolic labeling of newly synthesized LPS from *Salmonella*

In *Salmonella*, galactose is present in the LPS core as an α 1,3-linked backbone sugar and α 1,6-linked branch substituent [44,45]. As a result, *galE*-deficient *Salmonella* produces a truncated LPS when grown in the absence of galactose. However, the addition of galactose to the culture medium of *galE*-deficient *Salmonella* can restore the synthesis of the full-length backbone core and O-antigen of LPS, making it relatively easy to detect successful incorporation of azido-galactose variants. Fig. 2 exemplifies this by comparing the LPS composition from two *galE*-deficient strains of *Salmonella* (SF1195 and Ty21a) grown in the absence and presence of galactose. LPS from these two bacteria showed higher migrating bands and clear O-antigen ladders on SDS-PAGE only in the presence of galactose in both rich and minimal media (Fig. 2). There was no obvious advantage in growing the bacteria in a minimal medium. On the contrary, the overall amount and the amount of O-antigen containing LPS was significantly lower when the cells were grown in minimal media (compare 2B lanes 9 and 12). We think the varying production of LPS across different media can be attributed to the diverse nutrient compositions [46,47]. Thus, we only used LB from here on. Interestingly, a difference in O-antigen composition/length distribution could be observed between the two different *Salmonella* strains when grown in LB with galactose (compare Fig. 2A lane 5 and 2B lane 9). We reason that the chemical structure of the O-antigen, e.g. variation in the amount of repeating units, is highly variable among different strains [48]. Next, we tested if strain SF1195 could incorporate a galactose variant with an azido group at the 2, 4, and 6 positions into its LPS. We did not detect clear incorporation of the three azido-galactose variants into the LPS of SF1195 (Fig. 2B) even at increased concentrations of azido galactose and growth time (Fig. 2B).

Ty21a gave similar negative results when grown with 4- and 6-azido galactose, yet, a faint but readily detectable new band could be detected when we analyzed the LPS of this strain grown in the presence of 0.2 % 2-azido-2-deoxy-D-galactose (Fig. 3A, lane 3). Over-staining of the LPS in the gel made this LPS band a bit clear (Fig. 2C). Increasing the concentration of 2-azido-2-deoxy-D-galactose to 2 % and prolongation of the growth time from 8 to 18 h (Fig. 3B, lane 7) rendered this new band much more pronounced next to the appearance of other bands with lower mobility and even an O-antigen ladder similar to the 0.2 % galactose control. This indicated the successful incorporation of 2-azido-galactose into LPS by the cells. The 2-position is occupied by the azido group in 2-azido-2-deoxy galactose that is present in LPS of *Salmonella enterica* serovar Typhimurium, so other monosaccharides within the LPS structure cannot bond with 2-azido-2-deoxy galactose at its 2-position. Previous studies argued that the galactose in LPS of *Salmonella enterica* serovar Typhimurium is bonded through α -1,2 linkages or α -1,3 linkages [49–52]. Therefore, in conjunction with the previous reports, our results furthermore revealed that the *GalE* Ty21a strain's LPS contains galactoses that are connected via α -1,3 linkages as were suggested previously for *Salmonella enterica* serovar Typhimurium [52].

3.3. Time dependency of the incorporation of 2-azido-2-deoxy-galactose into LPS

Next, we examined the time dependency of the incorporation of azido galactose by labeling bacteria grown in the presence of 0.5 % 2-azido-2-deoxy galactose at different time points with a fluorescent probe using copper-free click chemistry with DBCO-Cy5. Analysis of the LPS with SDS-PAGE followed by in-gel fluorescence imaging revealed that, a half-hour after the addition of 2-azido-2-deoxy galactose, a fluorescent ladder of bands appeared from the top to bottom of the gel (Fig. 3C). The intensity of the fluorescent bands increased over time and seemed to reach a maximum level after about 8 h, where the most fluorescence was present in the lower 3 bands. We observed a difference in the patterns of LPS bands between silver-staining SDS-PAGE and in-gel fluorescent SDS-PAGE, which is likely caused by a difference in

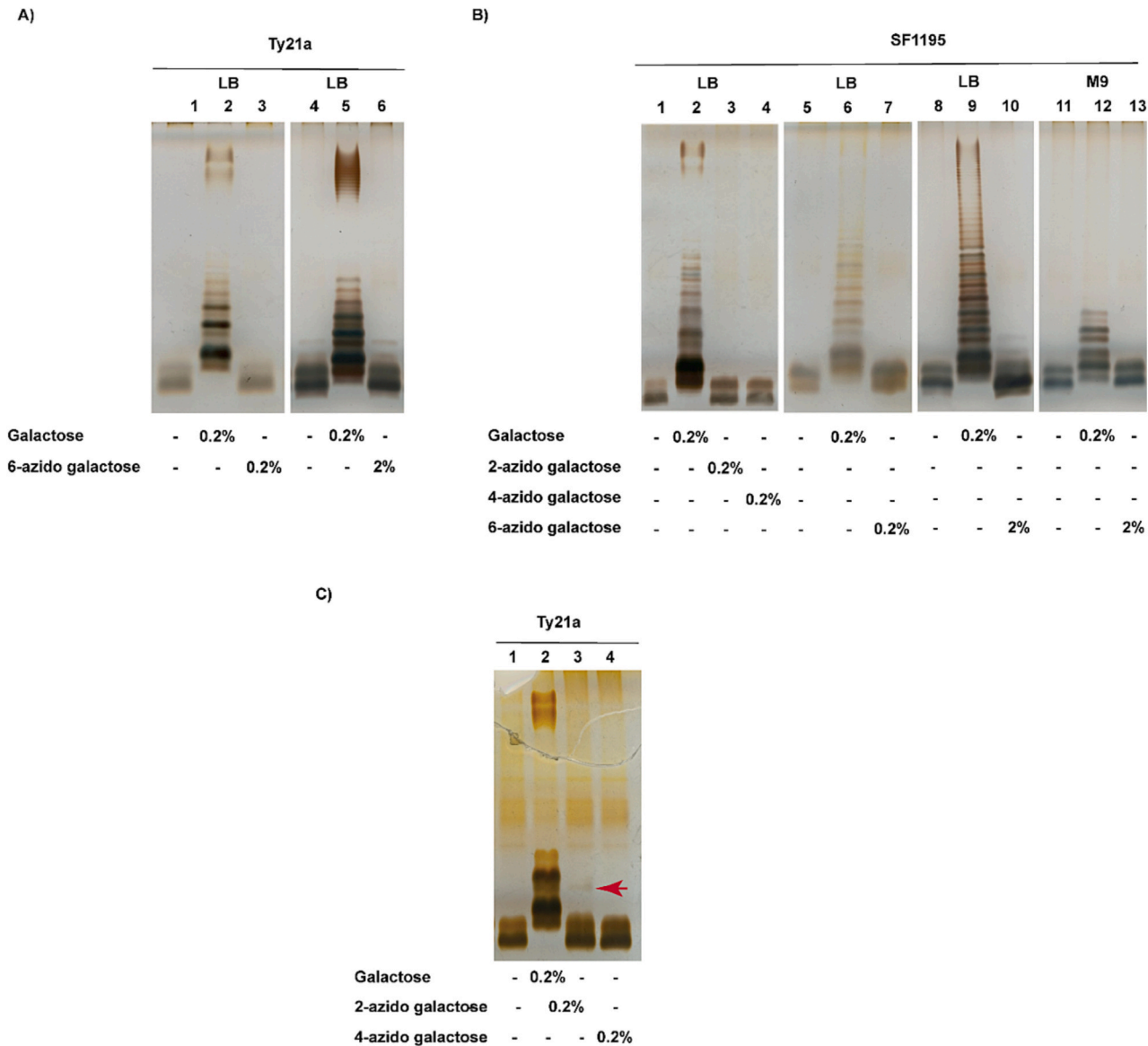


Fig. 2. Silver-staining SDS-PAGE of LPS isolated from SF1195 or Ty21a grown with galactose or azido-galactose. All of the LPS samples were prepared by removing protein with proteinase K. A) Silver-staining SDS-PAGE of LPS samples prepared from Ty21a. The bacteria were grown in LB with or without (denoted by the symbol “-”) galactose or 6-azido-6-deoxy galactose at different concentrations (0.2 % or 2 %) at 37 °C for 8 h in a shaker. B) Silver-staining SDS-PAGE of LPS samples prepared from SF1195. The bacteria were grown in LB or M9 (containing 0.2 % glucose) with or without different kinds of azido galactose (0.2 % or 2 %) at 37 °C for 18 h in a shaker. C) Time course analysis of galactose incorporation into Ty21a. LPS samples were prepared from the bacteria after they were incubated in LB with 0.2 % galactose at different time points and analyzed using silver-staining SDS-PAGE.

sensitivity of detection. Additionally, we found that there is less fluorescence in the O-antigen region. Apparently, the synthesis of the O-antigen and its coupling to the LPS core is more affected by the presence of the azide group than the synthesis of the LPS core (see also Fig. 3A). This is likely due to the differences in substrate specificity of the enzymes involved in the synthesis pathways. To elaborate, in the O-antigen of *Salmonella enterica* serovar Typhi LPS, galactose is linked with three other sugars through chemical bonds. Conversely, in the core of the LPS, galactose is only linked with one or two other galactose molecules (Fig. 1). As a result, incorporating galactose into the O-antigen of the LPS is more challenging than incorporating it into the LPS core. This difficulty arises because the incorporation of galactose into the O-antigen involves the participation of more enzymes compared to the incorporation into the LPS core. Consequently, the substrate specificity of these enzymes may lead to more hindrances in the incorporation of galactose into the O-antigen than in the core, resulting in fewer O-antigens being

labeled with azide.

3.4. Visualization of newly synthesized LPS in live bacteria

Since Ty21a can incorporate 2-azido-2-deoxy galactose into their LPS upon the addition of the azido-galactose to the medium, this method can in principle be used to track the appearance of newly synthesized LPS on the surface of bacteria. We first screened several clickable probes for fluorescent labeling of LPS and analyzed the labeled LPS with SDS-PAGE, which showed that copper-catalyzed probes gave the best results (Fig. 4). Next, we grew cells on 2-azido-2-deoxy galactose for 8 h, labeled them with Alexa Fluor 488-alkyne followed by fluorescence microscopy. While LPS labeling was not observed in the absence of a ‘clickable’ galactose, those grown in the presence of azido-galactose were clearly fluorescently stained (Figs. 5, S3). The fluorescence was most evident around the bacterial periphery, indicating components

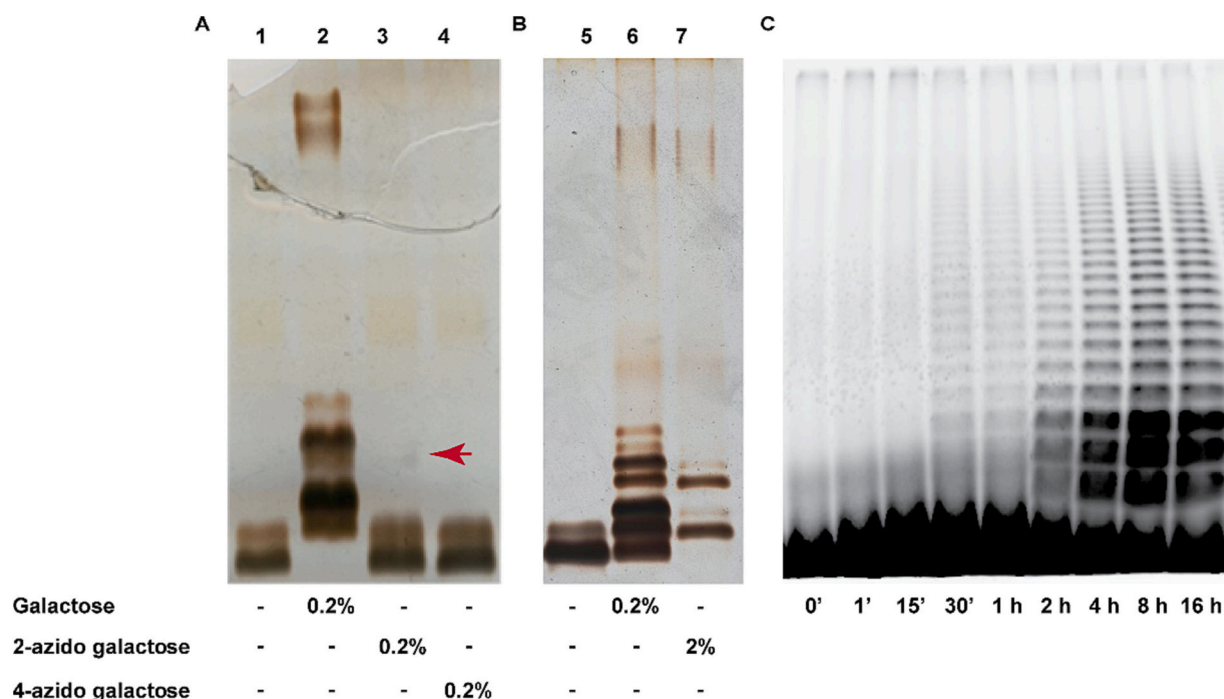


Fig. 3. Azido-galactose incorporation into LPS of Ty21a. A) SDS-PAGE gel analysis of LPS from Ty21a grown with different types of azido-galactoses visualized by silver-staining. The bacteria were grown in LB with or without 0.2 % galactose, 2-azido-2-deoxy galactose, or 4-azido-4-deoxy galactose at 37 °C for 8 h. Lanes: bacteria grown in LB only (1); or supplemented with 0.2 % galactose (2); with 0.2 % 2-azido-2-deoxy galactose (3); or with 0.2 % 4-azido galactose (4). The red arrow highlights the faint band that is visible only in lane 3. B) SDS-PAGE gel analysis of LPS from Ty21a grown in LB with or without 2 % 2-azido-2-deoxy galactose for 18 h visualized by silver-staining. Lanes: bacteria grown in LB only (5); in LB with 0.2 % galactose (6); in LB with 2 % 2-azido-2-deoxy galactose (7). C) Time-course analysis of 2-azido-2-deoxy galactose incorporation into the LPS of Ty21a. The LPS samples were incubated with DBCO-Cy5 and then imaged on a GE imager after being separated on an SDS-PAGE gel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

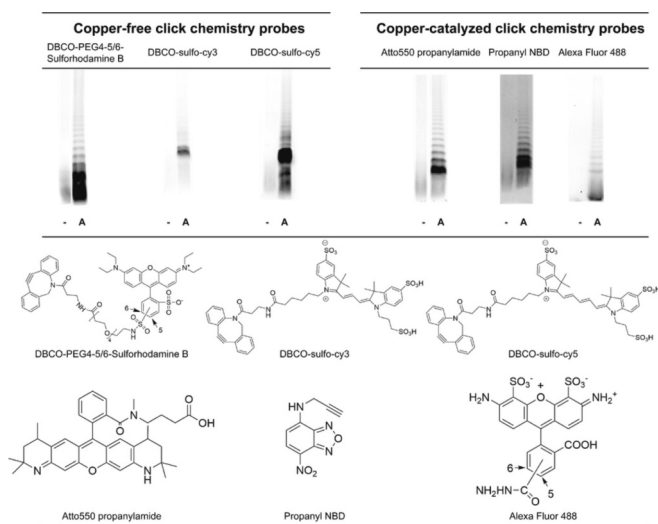


Fig. 4. Screening fluorescent copper-free click reagents and copper-catalyzed reagents for azido galactose labeling of Ty21a. Ty21a was grown in the medium with 2-azido-2-deoxy galactose (0.5 %), and LPS preparations from the bacterial cell were subsequently incubated with either copper-catalyzed click chemistry reagents (CuSO₄, 0.2 mM; THPTA, 1 mM; sodium ascorbic, 10 mM; probes, 25 μM) or copper-free click chemistry reagents (0.25 mM). The LPS samples were then analyzed by fluorescence scanning SDS-PAGE gels. “-”: medium without 2-azido-2-deoxy galactose, while “A”: medium with 2-azido-2-deoxy galactose (0.5 %).

that contain galactose on the bacterial cell surface were labeled. These labeled components are most likely azido galactose containing LPS. In principle, the labeled components could also include capsular

polysaccharides and bacterial glycoproteins in *Salmonella Typhi*. However, it's worth noting that the capsular polysaccharides of *S. typhi* consist of a linear polymer of α-(1 → 4) linked *N*-acetylgalactosaminuronic acid (GalNAcA) [53–55], making it improbable for azido galactose to be incorporated into these polysaccharides. Regarding glycoprotein labeling on bacterial surface, it's important to realize that most post-translational protein modifications occur in a relatively low number of bacterial proteins in comparison with eukaryotic proteins, and most of the modified proteins carry low, substoichiometric levels of modification [56]. Moreover, in *Salmonella enterica*, protein glycosylation typically occurs during bacterial infection, with the modification predominantly being GlcNAcylation [57,58]. Consequently, the possibility of protein modification and labeling with 2-azido-2-deoxy-galactose is minimal. All in all, the experimental results provide direct proof that the labeling on the surface of bacteria is attributed to LPS labeling.

Another interesting aspect that should be paid attention to is the location where the newly synthesized LPS was inserted. Previous studies have reported that the newly synthesized LPS is inserted into the OM in a stochastic manner, as observed in their labeling experiments [31–34]. During cell growth, insertion of newly synthesized LPS was proposed to primarily occur near the mid-cell rather than at the poles [31–34]. In label-and-chase experiments that were performed by these authors as well, newly inserted LPS near mid-cell pushes older clusters toward the poles, resulting in relatively intense fluorescence in the “old” poles due to the absence of growth in these regions after initial labeling. In our results, we observed denser fluorescence of newly synthesized LPS at the poles of certain bacteria compared to that near the mid-cell. These results are somewhat inconsistent with previously reported data and indicate that the insertion of newly synthesized LPS, at least in *Salmonella*, also occurs at the poles.

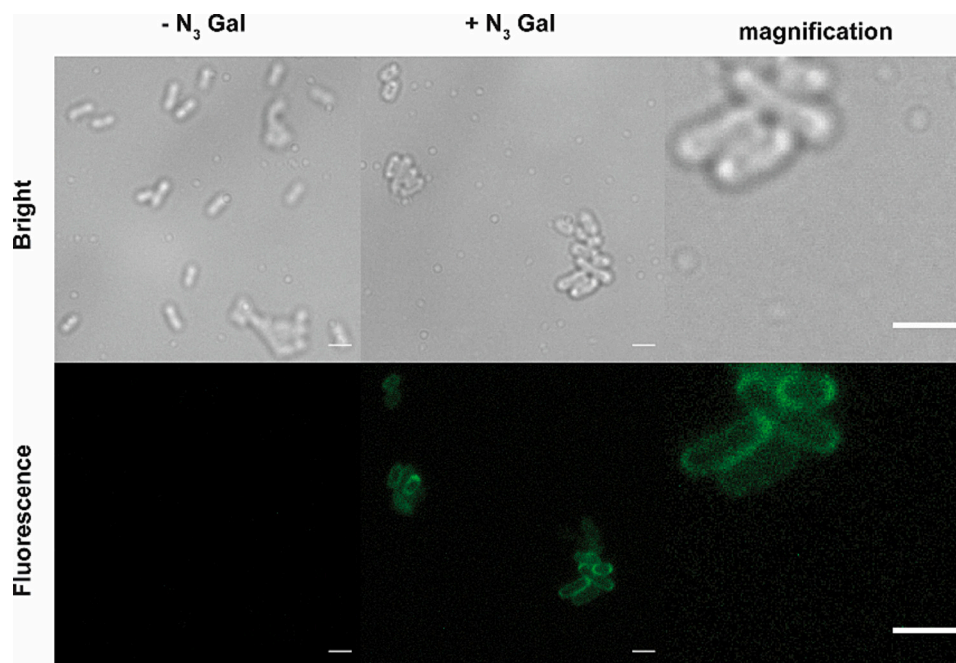


Fig. 5. Confocal microscopy image of Alexa Fluor 488-alkyne-labeled Ty21a grown with or without 2 % 2-azido-2-deoxy galactose. To prepare the sample, the Ty21a culture was allowed to grow overnight and then diluted 100-fold with fresh LB medium that contained 2-azido-2-deoxy galactose. The bacterial culture was grown in a shaker and incubated at 37 °C for 8 h. The metabolic incorporation of 2-azido-2-deoxy galactose in Ty21a was detected through a copper-catalyzed click reaction with the Alexa Fluor 488-alkyne. The middle panels of the figure present phase-contrast and fluorescence images of the bacteria grown in the presence of azido-galactose, while the left panels show the same for bacteria grown in its absence. The right panels show magnified images. The scale bar in the image is 2 μ m.

4. Conclusion

In our study, we have demonstrated that azido-galactose can be used as a chemical reporter for labeling newly synthesized lipopolysaccharides in actively growing bacteria, providing a novel approach for investigating the biosynthesis of lipopolysaccharide. The substrate specificity of enzymes involved in galactose metabolism and LPS biogenesis was a main determinant for the labeling efficiency of bacteria with different azido-functionalized galactoses. The tested bacterial strains showed varying capabilities for incorporating unnatural azido-galactoses. Moreover, the labeling method is also easily adaptable to other bacteria strains, provided that the substrate specificities of those strains allow the incorporation into LPS. This labeling strategy holds great potential for screening antibiotics that act at the level of the LPS biosynthesis pathway. By labeling bacteria with azido-galactose, it is possible to monitor the effects of antibiotics that target LPS in real time, as it allows the detection of newly synthesized LPS that appears on the OM surface. This approach can assist in the profiling and deconvolution of the mode of action of antibiotics.

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Yang Xu: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Xiaoqi Wang:** Methodology, Investigation. **Esther A. Zaai:** Methodology, Investigation, Data curation. **Celia R. Berkers:** Supervision, Methodology, Formal analysis. **Joseph H. Lorent:** Methodology, Formal analysis. **Torben Heise:** Writing – review & editing, Investigation. **Ruud Cox:** Supervision, Methodology. **Roland J. Pieters:** Writing – review & editing, Supervision, Formal

analysis. **Eefjan Breukink:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data reported in this paper will be shared by the lead contacts upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbalip.2024.159467>.

References

- [1] B. Liu Kenry, Bio-orthogonal click chemistry for in vivo bioimaging, *Trends Chem.* 1 (2019) 763–778, <https://doi.org/10.1016/j.trechm.2019.08.003>.
- [2] B. Imperiali, Bacterial carbohydrate diversity - a brave new world, *Curr. Opin. Chem. Biol.* 53 (2019) 1–8, <https://doi.org/10.1016/j.cbpa.2019.04.026>.
- [3] H.C. Hang, C. Yu, D.L. Kato, C.R. Bertozzi, A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 14846–14851, <https://doi.org/10.1073/pnas.2335201100>.
- [4] D.J. Vocadlo, H.C. Hang, E.J. Kim, J.A. Hanover, C.R. Bertozzi, A chemical approach for identifying O-GlcNAc-modified proteins in cells, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 9116–9121, <https://doi.org/10.1073/pnas.1632821100>.
- [5] M. Sawa, T.L. Hsu, T. Itoh, M. Sugiyama, S.R. Hanson, P.K. Vogt, C.H. Wong, Glycoproteomic probes for fluorescent imaging of fucosylated glycans in vivo, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 12371–12376, <https://doi.org/10.1073/pnas.0605418103>.
- [6] D. Rabuka, S.C. Hubbard, S.T. Laughlin, S.P. Argade, C.R. Bertozzi, A chemical reporter strategy to probe glycoprotein fucosylation, *J. Am. Chem. Soc.* 128 (2006) 12078–12079, <https://doi.org/10.1021/ja064619y>.
- [7] S.T. Laughlin, J.M. Baskin, S.L. Amacher, C.R. Bertozzi, In vivo imaging of membrane-associated glycans in developing zebrafish, *Science* 320 (2008) 664–667, <https://doi.org/10.1126/science.1155106>.
- [8] S.T. Laughlin, C.R. Bertozzi, In vivo imaging of Caenorhabditis elegans glycans, *ACS Chem. Biol.* 4 (2009) 1068–1072, <https://doi.org/10.1021/cb900254y>.
- [9] B.J. Beahm, K.W. Dehnert, N.L. Derr, J. Kuhn, J.K. Eberhart, D. Spillmann, S. L. Amacher, C.R. Bertozzi, A visualizable chain-terminating inhibitor of glycosaminoglycan biosynthesis in developing zebrafish, *Angew. Chem. Int. Ed. Engl.* 53 (2014) 3347–3352, <https://doi.org/10.1002/anie.201310569>.
- [10] R. Xie, L. Dong, Y. Du, Y. Zhu, R. Hua, C. Zhang, X. Chen, In vivo metabolic labeling of sialoglycans in the mouse brain by using a liposome-assisted bioorthogonal reporter strategy, *Proc. Natl. Acad. Sci. U. S. A.* 113 (2016) 5173–5178, <https://doi.org/10.1073/pnas.1516524113>.
- [11] N. Darabedian, J. Gao, K.N. Chuh, C.M. Woo, M.R. Pratt, The metabolic chemical reporter 6-Azido-6-deoxy-glucose further reveals the substrate promiscuity of O-GlcNAc transferase and catalyzes the discovery of intracellular protein modification by O-glucose, *J. Am. Chem. Soc.* 140 (2018) 7092–7100, <https://doi.org/10.1021/jacs.7b13488>.
- [12] R. Sadamoto, T. Matsubayashi, M. Shimizu, T. Ueda, S. Koshida, T. Koda, S. Nishimura, Bacterial surface engineering utilizing glucosamine phosphate derivatives as cell wall precursor surrogates, *Chemistry* 14 (2008) 10192–10195, <https://doi.org/10.1002/chem.200801734>.
- [13] H. Liang, K.E. DeMeester, C.W. Hou, M.A. Parent, J.L. Caplan, C.L. Grimes, Metabolic labelling of the carbohydrate core in bacterial peptidoglycan and its applications, *Nat. Commun.* 8 (2017) 15015, <https://doi.org/10.1038/ncomms15015>.
- [14] A. Dumont, A. Malleron, M. Awwad, S. Dukan, B. Vauzeilles, Click-mediated labeling of bacterial membranes through metabolic modification of the lipopolysaccharide inner core, *Angew. Chem. Int. Ed. Engl.* 51 (2012) 3143–3146, <https://doi.org/10.1002/anie.201108127>.
- [15] J. Mas Pons, A. Dumont, G. Sautejeau, E. Eugier, A. Baron, S. Dukan, B. Vauzeilles, Identification of living legionella pneumophila using species-specific metabolic lipopolysaccharide labeling, *Angew. Chem. Int. Ed. Engl.* 53 (2014) 1275–1278, <https://doi.org/10.1002/anie.201309072>.
- [16] I. Nilsson, K. Grove, D. Dovala, T. Uehara, G. Lapointe, D.A. Six, Molecular characterization and verification of azido-3,8-dideoxy-d-manno-oct-2-ulonic acid incorporation into bacterial lipopolysaccharide, *J. Biol. Chem.* 292 (2017) 19840–19848, <https://doi.org/10.1074/jbc.M117.814962>.
- [17] W. Yi, X. Liu, Y. Li, J. Li, C. Xia, G. Zhou, W. Zhang, W. Zhao, X. Chen, P.G. Wang, Remodeling bacterial polysaccharides by metabolic pathway engineering, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 4207–4212, <https://doi.org/10.1073/pnas.0812432106>.
- [18] G. Andolina, R. Wei, H. Liu, Q. Zhang, X. Yang, H. Cao, S. Chen, A. Yan, X.D. Li, X. Li, Metabolic labeling of Pseudaminic acid-containing Glycans on bacterial surfaces, *ACS Chem. Biol.* 13 (2018) 3030–3037, <https://doi.org/10.1021/acscchembio.8b00822>.
- [19] T. Heise, J.D. Langereis, E. Rossing, M.I. de Jonge, G.J. Adema, C. Büll, T.J. Boltje, Selective inhibition of sialic acid-based molecular mimicry in *Haemophilus influenzae* abrogates serum resistance, *Cell, Chem. Biol.* 25 (2018) 1279–1285, <https://doi.org/10.1016/j.cchembio.2018.05.018>.
- [20] A. Hoare, M. Bittner, J. Carter, S. Alvarez, M. Zaldívar, D. Bravo, M.A. Valvano, I. Contreras, The outer core lipopolysaccharide of *Salmonella enterica* serovar Typhi is required for bacterial entry into epithelial cells, *Infect. Immun.* 74 (2006) 1555–1564, <https://doi.org/10.1128/iai.74.3.1555-1564.2006>.
- [21] B.W. Simpson, M.S. Trent, Pushing the envelope: LPS modifications and their consequences, *Nat. Rev. Microbiol.* 17 (2019) 403–416, <https://doi.org/10.1038/s41579-019-0201-x>.
- [22] F. Di Lorenzo, K.A. Duda, R. Lanzetta, A. Silipo, C. De Castro, A. Molinaro, A journey from structure to function of bacterial lipopolysaccharides, *Chem. Rev.* (2021), <https://doi.org/10.1021/acs.chemrev.0c01321>.
- [23] A. Ebbensgaard, H. Mordhorst, F.M. Aarestrup, E.B. Hansen, The role of outer membrane proteins and lipopolysaccharides for the sensitivity of *Escherichia coli* to antimicrobial peptides, *Front. Microbiol.* 9 (2018) 2153, <https://doi.org/10.3389/fmicb.2018.02153>.
- [24] B. Bertani, N. Ruiz, Function and biogenesis of lipopolysaccharides, *EcoSal Plus* 8 (2018), <https://doi.org/10.1128/ecosalplus.ESP-0001-2018>.
- [25] C.A. Schnaitman, E.A. Austin, Efficient incorporation of galactose into lipopolysaccharide by *Escherichia coli* K-12 strains with polar *galE* mutations, *J. Bacteriol.* 172 (1990) 5511–5513, <https://doi.org/10.1128/jb.172.9.5511-5513.1990>.
- [26] S.A. Yoon, S.Y. Park, Y. Cha, L. Gopala, M.H. Lee, Strategies of detecting bacteria using fluorescence-based dyes, *Front. Chem.* 9 (2021) 743923, <https://doi.org/10.3389/fchem.2021.743923>.
- [27] M. Grabowicz, D. Andres, M.D. Lebar, G. Malojčić, D. Kahne, T.J. Silhavy, A mutant *Escherichia coli* that attaches peptidoglycan to lipopolysaccharide and displays cell wall on its surface, *Elife* 3 (2014) e05334, <https://doi.org/10.7554/eLife.05334>.
- [28] S. Aymanns, S. Mauere, G. van Zandbergen, C. Wolz, B. Spellerberg, High-level fluorescence labeling of gram-positive pathogens, *PLoS One* 6 (2011) e19822, <https://doi.org/10.1371/journal.pone.0019822>.
- [29] T. Mohammadi, V. van Dam, R. Sijbrandi, T. Vernet, A. Zapun, A. Bouhss, M. Diepeveen-de Bruin, M. Nguyen-Distèche, B. de Kruijff, E. Breukink, Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane, *EMBO J.* 30 (2011) 1425–1432, <https://doi.org/10.1038/emboj.2011.61>.
- [30] H.M. Holden, I. Rayment, J.B. Thoden, Structure and function of enzymes of the Leloir pathway for galactose metabolism, *J. Biol. Chem.* 278 (2003) 43885–43888, <https://doi.org/10.1074/jbc.R300025200>.
- [31] T.S. Ursell, E.H. Trepagnier, K.C. Huang, J.A. Theriot, Analysis of surface protein expression reveals the growth pattern of the gram-negative outer membrane, *PLoS Comput. Biol.* 8 (2012) e1002680, <https://doi.org/10.1371/journal.pcbi.1002680>.
- [32] P. Rassam, N.A. Copeland, O. Birkholz, C. Tóth, M. Chavent, A.L. Duncan, S. J. Cross, N.G. Housden, R. Kaminska, U. Seger, D.M. Quinn, T.J. Garrod, M. S. Sansom, J. Piehler, C.G. Baumann, C. Kleanthous, Supramolecular assemblies underpin turnover of outer membrane proteins in bacteria, *Nature* 523 (2015) 333–336, <https://doi.org/10.1038/nature14461>.
- [33] I. Botos, N. Noinaj, S.K. Buchanan, Insertion of proteins and lipopolysaccharide into the bacterial outer membrane, *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 372 (2017), <https://doi.org/10.1098/rstb.2016.0224>.
- [34] J. Sun, S.T. Rutherford, T.J. Silhavy, K.C. Huang, Physical properties of the bacterial outer membrane, *Nat. Rev. Microbiol.* 20 (2022) 236–248, <https://doi.org/10.1038/s41579-021-00638-0>.
- [35] P.F. Mühlradt, J.R. Golecki, Asymmetrical distribution and artifactual reorientation of lipopolysaccharide in the outer membrane bilayer of *Salmonella typhimurium*, *Eur. J. Biochem.* 51 (1975) 343–352, <https://doi.org/10.1111/j.1432-1033.1975.tb03934.x>.
- [36] R. Germanier, E. Frier, Isolation and characterization of Gal E mutant Ty 21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine, *J. Infect Dis* 131 (1975) 553–558, <https://doi.org/10.1093/infdis/131.5.553>.
- [37] N.N. Nickerson, L.L. Mainprize, L. Hampton, M.L. Jones, J.H. Naismith, C. Whitfield, Trapped translocation intermediates establish the route for export of capsular polysaccharides across *Escherichia coli* outer membranes, *Proc. Natl. Acad. Sci. U. S. A.* 111 (2014) 8203–8208, <https://doi.org/10.1073/pnas.1400341111>.
- [38] R.B. Snitynsky, T.L. Lowary, Synthesis of nitrogen-containing furanose sugar nucleotides for use as enzymatic probes, *Org. Lett.* 16 (2014) 212–215, <https://doi.org/10.1021/ol4032073>.
- [39] H. Ye, R. Liu, D. Li, Y. Liu, H. Yuan, W. Guo, L. Zhou, X. Cao, H. Tian, J. Shen, P. G. Wang, A safe and facile route to imidazole-1-sulfonyl azide as a diazotransfer reagent, *Org. Lett.* 15 (2013) 18–21, <https://doi.org/10.1021/ol3028708>.
- [40] R. Kittelberger, F. Hilbink, Sensitive silver-staining detection of bacterial lipopolysaccharides in polyacrylamide gels, *J. Biochem. Biophys. Methods* 26 (1993) 81–86, [https://doi.org/10.1016/0165-022x\(93\)90024-j](https://doi.org/10.1016/0165-022x(93)90024-j).
- [41] J. Yang, X. Fu, Q. Jia, J. Shen, J.B. Biggins, J. Jiang, J. Zhao, J.J. Schmidt, P. G. Wang, J.S. Thorson, Studies on the substrate specificity of *Escherichia coli* galactokinase, *Org. Lett.* 5 (2003) 2223–2226, <https://doi.org/10.1021/ol034642d>.
- [42] M. Chen, L.L. Chen, Y. Zou, M. Xue, M. Liang, L. Jin, W.Y. Guan, J. Shen, W. Wang, L. Wang, J. Liu, P.G. Wang, Wide sugar substrate specificity of galactokinase from *Streptococcus pneumoniae* TIGR4, *Carbohydr. Res.* 346 (2011) 2421–2425, <https://doi.org/10.1016/j.carres.2011.08.014>.
- [43] Y. Zou, W. Wang, L. Cai, L. Chen, M. Xue, X. Zhang, J. Shen, M. Chen, Substrate specificity of galactokinase from *Streptococcus pneumoniae* TIGR4 towards galactose, glucose, and their derivatives, *Bioorg. Med. Chem. Lett.* 22 (2012) 3540–3543, <https://doi.org/10.1016/j.bmcl.2012.03.095>.
- [44] H. Nikaido, Studies on the biosynthesis of cell wall polysaccharide in mutant strains of *Salmonella*, II, *Proc. Natl. Acad. Sci. U. S. A.* 48 (1962) 1542–1548, <https://doi.org/10.1073/pnas.48.9.1542>.
- [45] M.J. Osborn, S.M. Rosen, L. Rothfield, B.L. Horecker, Biosynthesis of bacterial lipopolysaccharide. I. Enzymatic incorporation of galactose in a mutant strain of *Salmonella*, *Proc. Natl. Acad. Sci. U. S. A.* 48 (1962) 1831–1838, <https://doi.org/10.1073/pnas.48.10.1831>.
- [46] G. Zhang, T.C. Meredith, D. Kahne, On the essentiality of lipopolysaccharide to gram-negative bacteria, *Curr. Opin. Microbiol.* 16 (2013) 779–785, <https://doi.org/10.1016/j.mib.2013.09.007>.
- [47] E.J. McGroarty, M. Rivera, Growth-dependent alterations in production of serotype-specific and common antigen lipopolysaccharides in *Pseudomonas aeruginosa* PAO1, *Infect. Immun.* 58 (1990) 1030–1037, <https://doi.org/10.1128/iai.58.4.1030-1037.1990>.
- [48] B. Liu, Y.A. Knirel, L. Feng, A.V. Perepelov, S.N. Senchenkova, P.R. Reeves, L. Wang, Structural diversity in *Salmonella* O antigens and its genetic basis, *FEMS Microbiol. Rev.* 38 (2014) 56–89, <https://doi.org/10.1111/1574-6976.12034>.

- [49] J. Qian, T.A. Garrett, C.R. Raetz, In vitro assembly of the outer core of the lipopolysaccharide from *Escherichia coli* K-12 and *Salmonella typhimurium*, *Biochemistry* 53 (2014) 1250–1262, <https://doi.org/10.1021/bi4015665>.
- [50] D.E. Heinrichs, J.A. Yethon, C. Whitfield, Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*, *Mol. Microbiol.* 30 (1998) 221–232, <https://doi.org/10.1046/j.1365-2958.1998.01063.x>.
- [51] M.M. Olsthoorn, B.O. Petersen, J. Duus, J. Haverkamp, J.E. Thomas-Oates, K. Bock, O. Holst, The structure of the linkage between the O-specific polysaccharide and the core region of the lipopolysaccharide from *Salmonella enterica* serovar typhimurium revisited, *Eur. J. Biochem.* 267 (2000) 2014–2027, <https://doi.org/10.1046/j.1432-1327.2000.01205.x>.
- [52] J.R. Littlejohn, R.F. da Silva, W.A. Neale, C.C. Smallcombe, H.W. Clark, R. A. Mackay, A.S. Watson, J. Madsen, D.W. Hood, I. Burns, T.J. Greenhough, A. K. Shrive, Structural definition of hSP-D recognition of *Salmonella enterica* LPS inner core oligosaccharides reveals alternative binding modes for the same LPS, *PLoS One* 13 (2018) e0199175, <https://doi.org/10.1371/journal.pone.0199175>.
- [53] K. Heyns, G. Kiessling, Strukturaufklärung des vi-antigens aus *Citrobacter freundii* (*E. coli*) 5396/38, *Carbohydr. Res.* 3 (1967) 340–353, [https://doi.org/10.1016/S0008-6215\(00\)82210-7](https://doi.org/10.1016/S0008-6215(00)82210-7).
- [54] E.M. Daniels, R. Schneerson, W.M. Egan, S.C. Szu, J.B. Robbins, Characterization of the *Salmonella paratyphi* C Vi polysaccharide, *Infect. Immun.* 57 (1989) 3159–3164, <https://doi.org/10.1128/iai.57.10.3159-3164.1989>.
- [55] S.S. Wear, C. Sande, O.G. Ovchinnikova, A. Preston, C. Whitfield, Investigation of core machinery for biosynthesis of Vi antigen capsular polysaccharides in gram-negative bacteria, *J. Biol. Chem.* 298 (2022) 101486, <https://doi.org/10.1016/j.jbc.2021.101486>.
- [56] B. Macek, K. Forchhammer, J. Hardouin, E. Weber-Ban, C. Grangeasse, I. Mijakovic, Protein post-translational modifications in bacteria, *Nat. Rev. Microbiol.* 17 (2019) 651–664, <https://doi.org/10.1038/s41579-019-0243-0>.
- [57] J. Xue, Y. Huang, H. Zhang, J. Hu, X. Pan, T. Peng, J. Lv, K. Meng, S. Li, Arginine GlcNAcylation and activity regulation of PhoP by a type III secretion system effector in *Salmonella*, *Front. Microbiol.* 12 (2021) 825743, <https://doi.org/10.3389/fmicb.2021.825743>.
- [58] X. Pan, J. Luo, S. Li, Bacteria-catalyzed arginine glycosylation in pathogens and host, *Front. Cell. Infect. Microbiol.* 10 (2020) 185, <https://doi.org/10.3389/fcimb.2020.00185>.